

***ATM* COPY NUMBER LOSS AS A BIOMARKER FOR RADIORESISTANCE IN
UROTHELIAL BLADDER CANCER CELL LINES**

by

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ABSTRACT

Cancer is one of the leading causes of death in the United States today. Various techniques have been adopted to combat this disease, including chemotherapy and ionizing radiation therapy. Previous studies have suggested that certain cancer cells are more likely to survive when exposed to ionizing radiation, a phenomenon known as radioresistance. In oral squamous cell carcinoma, one biomarker for radioresistance is the copy number loss of distal 11q (marked by the *ATM* gene) and defective TP53 function. Our study is designed to determine whether copy number loss of *ATM* is linked with radioresistance instead of the combination of distal 11q loss and defective TP53 function. Based on clonogenic survival data, we hypothesize that other factors including miR100 and/or *SMARCA5* should be investigated as potential predictors of radioresistance.

Public Health Significance: Cancer is a widespread public health problem. The goal of this research is to identify targets that lead to increased therapeutic efficacy. In light of our results, further studies can be designed to establish a more accurate biomarker for radioresistance in urothelial bladder cancer cell lines. Further studies should investigate the roles of miR100 and *SMARCA5* in radioresistance in these cell lines. In essence, this research study shows that the *ATM* gene may not be the best predictor of radioresistance in bladder cancer cell lines, but perhaps a combination of events can explain our findings.

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PREFACE

It is with immense gratitude that I acknowledge the support and help of my advisor, Dr. Susanne M. Gollin. Without her expert guidance and persistence throughout the entirety of the project, this thesis would not have been possible.

I would like to thank Drs. Michael Barmada and Rahul Parikh for their time in the evaluation process and for helpful advice on the project.

I consider it an honor to have worked with Dale W. Lewis, Elizabeth Lawrence, and all of the Human Genetics Laboratory personnel. Their advice on techniques and troubleshooting was invaluable.

I would like to thank the Learmonth Fund of The Pittsburgh Foundation for funding that made this project possible.

I owe my deepest gratitude to my family. Without their words of encouragement and moral guidance, I would not have achieved this goal.

1.0 INTRODUCTION

Cancer is a widespread public health problem in the global community. It is estimated that nearly one in four deaths in the United States is cancer-related (Siegel et al., 2015). In general, cancer is caused by the loss of tumor suppressor genes and the gain or amplification of oncogenes. A subset of these genes plays a crucial role in regulating cell cycle pathways. The end result of defects in these genes is a cell that exhibits dysregulated cellular proliferation.

To combat cancer, scientists have attempted to devise effective ways of killing cancer cells before they can grow in number and spread throughout the body. The most common methods to combat these cells are chemotherapy and ionizing radiation (IR) therapy. The mechanism underlying these therapies is to create sufficient DNA damage that the cells ultimately die due to various processes such as mitotic catastrophe, apoptosis, and/or necrosis.

Tumor suppressor genes and oncogenes are not the only genes that are altered in cancer cells. Typically, cancers also have defects in their DNA damage response pathways. Recent studies in oral squamous cell carcinomas (OSCC) have suggested that certain defects, specifically copy number loss of distal chromosome 11q as marked by the *ATM* (Ataxia Telangiectasia Mutated) gene and defective TP53 function, lead to an increase in survival of cancer cells after treatment with radiation therapy (Parikh et al., 2007; Sankunny et al., 2014).

To compensate for the copy number loss of *ATM*, the ATR-CHEK1 pathway becomes overexpressed (Sankunny et al., 2014). This pathway has been linked with increased G₂M

cellular arrest (Sankunny et al., 2014). Under these circumstances, the cells become arrested in the G₂M phase of the cell cycle long enough to repair the damage and subsequently continue dividing. For this reason, investigators are attempting to normalize the expression pattern of either ATR or CHEK1 to resensitize these cells to ionizing radiation.

In previous studies from our laboratory, all of the cell lines that showed radioresistance had both copy number loss of *ATM*, as well as defective TP53 signaling. It is unclear, however, if radioresistance can be accurately predicted by only one of these conditions rather than both. In this study, we seek to answer this question by using urothelial bladder cancer cell lines that have either a defective TP53 pathway with normal copy number (or copy number gain) of *ATM* or copy number loss of *ATM* and a fully functional TP53 pathway.

1.1 EPIDEMIOLOGY OF BLADDER CANCER

In 2015, it was estimated that there will be 1,658,370 new cases of all forms of cancer in just the United States alone (Siegel et al., 2015). The number of deaths resulting from cancer is expected to be 589,430 deaths (Siegel et al., 2015). Bladder cancer, however, only accounts for 4.46% of these new cases (or 74,000 new cases), and 16,000 deaths. (Siegel et al., 2015). The Cancer Genome Atlas estimates that nearly 25% of all cancers and 37% of all bladder cancers have copy number loss of *ATM* (TCGA, 2015). Therefore, if those bladder cancers are resistant to therapy, as many as 27,380 patients might be saved in the U.S. by resensitizing their tumors to therapy by inhibiting the ATR-CHEK1 pathway.

1.2 CHROMOSOMAL INSTABILITY AND AMPLIFICATION OF *CCND1*

Chromosomal instability is a phenomenon that is characterized by the loss or gain of whole segments of chromosomes in a population of cells. *CCND1* is a gene that codes for cyclin D1 and is located on chromosome 11 in band 11q13. One of the prevailing mechanisms underlying the amplification of *CCND1* is breakage-fusion-bridge cycles (Reshmi et al., 2007a). This mechanism occurs as follows. First, a break in the chromosome must occur (Reshmi et al., 2007a). As it relates to *CCND1* amplification, this initial break happens distal to *CCND1* at 11q14 affecting a chromosome fragile site known as *FRA11F* (Reshmi et al., 2007b). After the initial break occurs, the two chromatids are considered to have double stranded breaks and are repaired by being ligated together at the breakage site, creating a dicentric chromosome (Reshmi et al., 2007a). After the fusion, a second break occurs upstream from the site (Reshmi et al., 2007a). This leads to an inverted duplication of the gene(s) proximal to the initial break, which in this case includes *CCND1* (Reshmi et al., 2007a). However, this mechanism results in the loss of downstream genes. In the case of *CCND1* amplification, distal 11q is often lost (Reshmi et al., 2007a). A set of DNA damage response genes is located on distal 11q including *H2AFX*, *MRE11A*, *ATM*, and *CHEK1* (Parikh et al., 2007; Sankunny et al., 2014). Given that this mechanism is associated with loss of distal 11q, and *ATM* is located distal to *CCND1*, it is reasonable to suspect that this mechanism may play some role in the copy number alterations of *ATM* in these cancer cells.

1.3 DNA DAMAGE RESPONSE TO DOUBLE STRANDED BREAKS: THE ATM PATHWAY

Damage to the genome is one of the hallmarks of a cancer cell (Hanahan and Weinberg, 2011). A type of DNA damage that is commonly seen is the double stranded break, or DSB. DSBs are typically caused by ionizing radiation, ultraviolet radiation, and chemical compounds (Jackson and Bartek, 2009). For this study, we shall focus on IR-induced DSBs.

With IR-induced DSBs, the MRE11A-RAD50-NBS1 (MRN) complex is signaled to the site of the DSB. This complex has been linked to the rapid phosphorylation of ATM (Carson et al., 2003). ATM, in turn, phosphorylates a variety of proteins related to cell cycle checkpoints, such as TP53 and CHEK2 (Tanaka et al., 2007). TP53 and CHEK2 effect cell cycle arrest in the G₁ phase (Tanaka et al., 2007). ATM also phosphorylates H2AFX (Tanaka et al., 2007). H2AFX is a histone that, when activated, is responsible for assisting in the attachment of DNA damage response proteins (Stucki et al., 2005). ATM has been hypothesized to play a role in suppressing chromosomal instability (White et al., 2008). With copy number loss of *ATM*, the ATR pathway has been shown to become overexpressed (Sankunny et al., 2014).

1.4 THE ATR PATHWAY

ATR is a gene that is located in chromosome band 3q23. This gene, like *ATM*, is involved in the DNA damage response pathway. Stalled replication forks typically lead to the rapid phosphorylation of the ATR protein (Huang et al, 2008). The ATR pathway leads to single stranded DNA which can then be repaired. DSBs have also been linked to creating single

stranded DNA through homologous recombination (Huertas, 2010). Given this concept, the ATR and ATM pathways may be highly intertwined rather than just parallel pathways.

ATR, when recruited to the site of the DSB, actively phosphorylates the CHEK1 protein at ser317 and ser345 (Huang et al, 2008). Upon phosphorylation, CHEK1 then phosphorylates CDC25 (Huang et al, 2008). Once phosphorylated, CDC25 is no longer able to activate CDK1, resulting in the arrest of the cells in the G₂M phase of the cell cycle (Wang et al., 2015). ATR or CHEK1 overexpression leads to a longer arrest in the G₂M phase, which has been shown to be linked with radioresistance (Sankunny et al., 2014).

1.5 THE PROJECT GOALS

Based on previous studies from our laboratory, radioresistance was seen in OSCC cancer cells that had distal 11q loss as marked by copy number loss of the *ATM* gene and defective TP53 function. For this study, we hypothesize that copy number loss of *ATM* is associated with radioresistance in urothelial bladder cancer cells rather than the combination of *ATM* and TP53 status. The goals of this study are to 1) demonstrate that urothelial bladder cancer cell lines have copy number loss of *ATM*, 2) show that these cell lines have either a functional or non-functional TP53, and 3) determine whether radioresistance can be predicted using only *ATM* copy number status.

2.0 MATERIALS AND METHODS

2.1 DATABASE MINING OF CELL LINE INFORMATION

To identify optimal cell lines for this study, copy number alterations for certain genes needed to be obtained before cell lines could be chosen. The UCSC Genome Browser was used in order to determine the position of certain genes on chromosome 11 (<http://genome.ucsc.edu/cgi-bin/hgGateway>). These genes included *ATM*, *CHEK1*, *MRE11A*, and *H2AFX*. A graph representing copy number based on SNPs on chromosome 11 was obtained from the Catalogue of Somatic Mutations in Cancer database (http://cancer.sanger.ac.uk/cell_lines). The locations of these genes were cross-referenced with this graph in order to determine the predicted copy number of these genes in various bladder cancer cell lines. *TP53* status was determined using the IARC *TP53* database (<http://p53.iarc.fr/CellLines.aspx>).

2.2 CELL CULTURE

We selected five cell lines that had either copy number loss or copy number gain/neutral copy number for *ATM* according to the Catalogue of Somatic Mutations in Cancer database (COSMIC). These five cell lines, HTB9, T24, SW1710, J82, and VM-CuB-1 were obtained

from ATCC or DSMZ. Cell lines HTB9, SW1710, and VM-CuB-1 were cultured in Dulbecco's Minimum Essential medium. Cell line J82 was cultured with Eagle's Minimum Essential medium. Cell line T24 was cultured in McCoy's 5A medium. All media were supplemented with 1% L-Glutamine, 1% non-essential amino acids (NEAA), 10% fetal bovine serum (FBS), and 0.05 mg/mL of Gentamicin. All cell lines were examined every two days using an Olympus inverted phase contrast microscope to determine confluency. The cell lines were then passaged if the confluency was greater than 70% after rinsing with Hanks Balanced Salts Solution (HBSS) and dissociating the cells with 0.05% trypsin-EDTA.

2.3 QUALITY ASSURANCE OF THE CELL LINES

To ensure that the cell lines used in this study are the correct cell lines provided to our laboratory, we chose to carry out STR profiling ("DNA Fingerprinting") of these cells. Total DNA was extracted from cultured cells at the beginning and end of the project using a Qiagen DNeasy Tissue Kit (Qiagen, MD). The DNA was then amplified using an AmpF STR Identifier PCR Kit (Applied Biosystems, NY). The STR profiles were determined, or "called", and verified using Gene Mapper version 4.0 (Applied Biosystems, NY). The "calls" were cross-referenced using STR profiling software provided online by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The STR profile at the beginning of the project was performed and analyzed by Dale W. Lewis of the University of Pittsburgh Cell Culture and Cytogenetics Facility. The STR profile at the end of the project was performed by Dale W. Lewis and analyzed by Chad H. Lawrence.

2.4 FLUORESCENCE IN SITU HYBRIDIZATION

We prepared cell lines for FISH by arresting them in metaphase using Colcemid for 1 hour at 37°C. The cells were then placed in hypotonic solution consisting of 0.075M potassium chloride (KCl) for 30 minutes at 37°C. The cells were fixed in Carnoy's fixative (3:1 methanol:glacial acetic acid) at room temperature and dropped onto slides. The slides were treated in 2x saline-sodium citrate (SSC) supplemented with RNase for 30 minutes at 37°C. Afterward, the slides were dehydrated using 70%, 80%, and 100% ethanol at room temperature. Cells on the slide were exposed to 70% formamide for 4 minutes at 75°C then dehydrated again using 70%, 80%, and 100% ethanol at room temperature. An *ATM* deletion probe (Cytocell, Cambridge, United Kingdom) was used. This probe detects both the *ATM* gene region (red) and the centromere of chromosome 11 (green). This probe was denatured at 75°C and allowed to reanneal at 37°C for 15-30 minutes. The probe was hybridized to the slide for 24 hours at 37°C. The slides were then washed with 2x SSC supplemented with Tween 20. DAPI (4',6-diamidino-2-phenylindole) was used to stain the chromatin of the cells. Copy number analysis was performed using an Olympus fluorescence microscope. Copy number alterations of *ATM* were based on the ratio between red and green signals. Copy number gain was defined as having a ratio greater than 1, while copy number loss was defined as having a ratio less than 1. Greater than 200 cells were counted for each cell line. Images of representative cells were captured using an Olympus BX61 fluorescence microscope and the GENUS software package (Leica Biosystems, IL).

2.5 WESTERN BLOTTING

The Western blotting technique was used to ascertain the TP53 status (functional or non-functional) of the aforementioned cell lines. One flask of each cell line was treated with Adriamycin (doxorubicin) for 4 hours. The flasks were then washed with cold (4°C) 1x Phosphate Buffered Saline (PBS) and the cells were removed using a cell scraper. The cells were lysed in a solution containing Tris Base, Triton x100, sodium dodecyl sulfate (SDS), NaCl, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF). The solution was centrifuged at 13,500xg for 30 minutes at 4°C. The pellet was resuspended in the lysis solution. The lysates were resolved using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was cut to allow the TP53 and p21 (CDKN1A) signals to show (as the secondary antibodies used to detect the primary antibodies were antagonistic). The membrane was left to dry. Afterwards, the membrane was blocked using Li-Cor TBS blocking buffer for 1 hour. The top membrane (containing the higher molecular weight protein TP53) was incubated at 4°C overnight with the antibody for TP53 (Santa Cruz 1:1000 dilution). The bottom membrane (containing the lower molecular weight proteins p21 and tubulin) was incubated at 4°C overnight with the antibodies p21 (1:1000 dilution; Santa Cruz) and Alpha-Beta Tubulin (1:1000 dilution; Santa Cruz). Four washes of 1x TBST (Tris Buffered Saline with Tween 20) were performed. The membranes were incubated at room temperature with the appropriate fluorescent secondary antibody specific to the animal source of the primary antibody at a dilution of 1:10,000 for each antibody for 1 hour. While protecting the membranes from light, they were washed four times with 1x TBST was performed followed by a wash of 1x TBS and water. The membranes were dried at room temperature in the dark for 1-2 hours. The target proteins were imaged on a Li-Cor Odyssey CLx infrared imaging system.

2.6 CLONOGENIC SURVIVAL ASSAYS

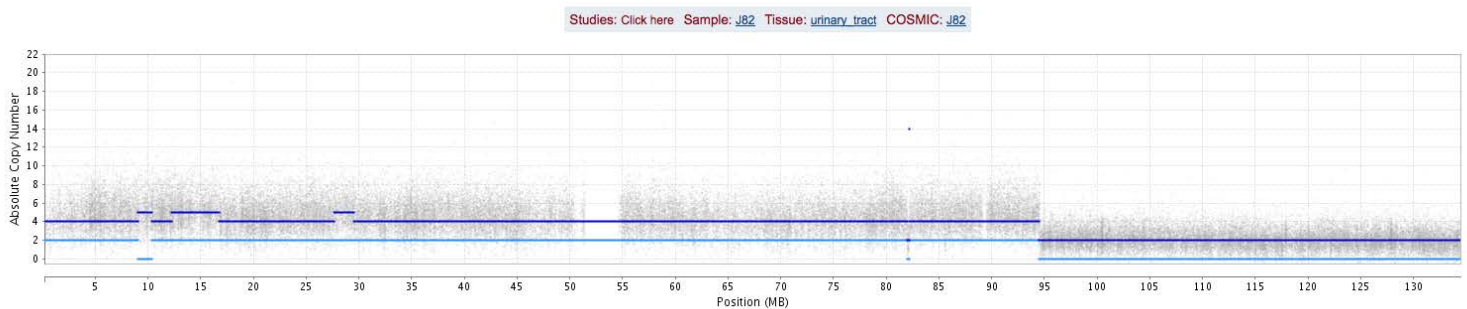
In order to determine radiosensitivity or survival of the cell lines after treatment with radiation, clonogenic (colony) survival assays were set up. The number of living cells in a cell suspension of the cell lines was assessed using trypan blue and a hemocytometer. Two thousand living cells were plated on a 60mm culture dish and were allowed to attach for 6 hours. The cells were then exposed to various levels of irradiation (0 Gy, 2.5 Gy, or 5 Gy). This process was performed in triplicate for each cell line at each treatment level. After 5 days, the culture medium was changed. The cells were grown until the colonies that formed were comprised of >50 cells. At this time, the cells were fixed in 70% ethanol and stained with Giemsa stain. Stained colonies were counted. Plating efficiency of the culture dishes was taken into account and was based on the untreated (mock-treated) dishes. Surviving fractions were then calculated based on plating efficiency and number of colonies.

3.0 RESULTS

3.1 DATABASE INQUIRY RESULTS

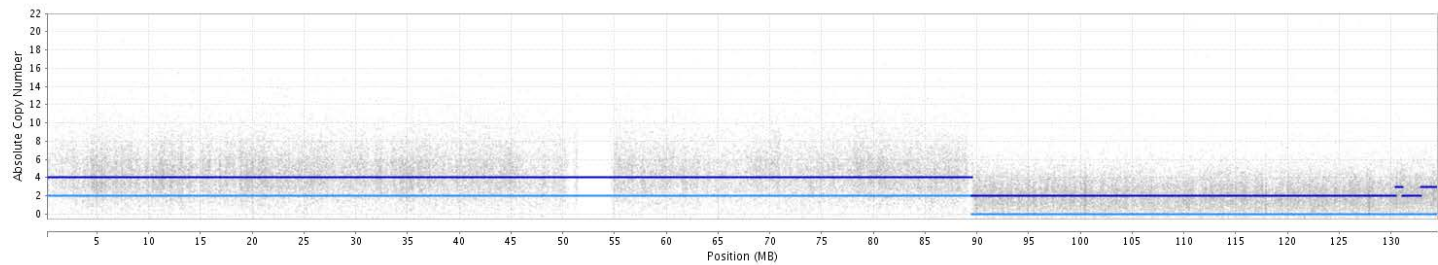
Various databases were consulted in order to determine *ATM* gene copy number in for various cell lines. These databases included IARC TP53, COSMIC, and the UCSC Genome Browser. Figure 1 shows the graphical representation of copy number as presented by COSMIC (2014) for all cell lines used except HTB9 (which is no longer available as of 2015 and can therefore not be shown). Table 1 summarizes the results of the database mining. From these results, we expected that VM-CuB-1, J82, and SW1710 would have copy number loss of *ATM*. T24 and HTB9 are suspected to have copy number gain or copy number neutral for *ATM*. TP53 is suspected to be mutated across all cell lines listed.

SNP Array Based LOH and Copy Number Analysis J82 (Chromosome 11)



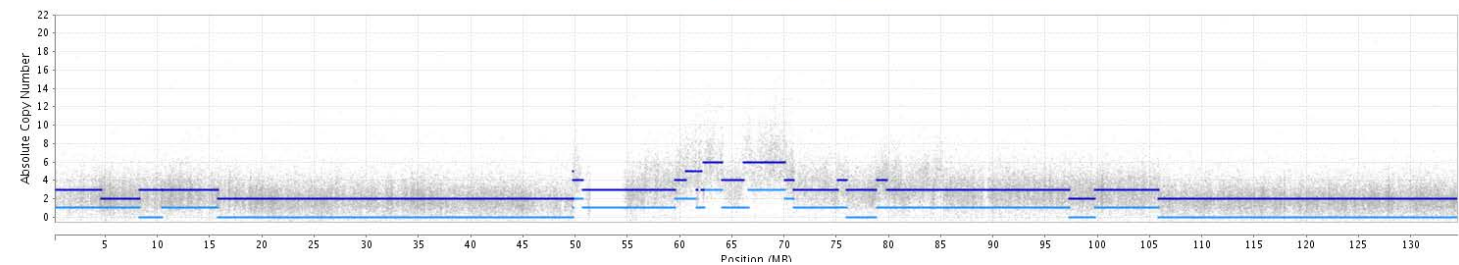
SNP Array Based LOH and Copy Number Analysis
SW1710 (Chromosome 11)

Studies: [Click here](#) Sample: [SW1710](#) Tissue: [urinary tract](#) COSMIC: [SW1710](#)



SNP Array Based LOH and Copy Number Analysis
VM-CUB-1 (Chromosome 11)

Studies: [Click here](#) Sample: [VM-CUB-1](#) Tissue: [urinary tract](#) COSMIC: [VM-CUB-1](#)



SNP Array Based LOH and Copy Number Analysis
T-24 (Chromosome 11)

Studies: [Click here](#) Sample: [T-24](#) Tissue: [urinary tract](#) COSMIC: [T-24](#)

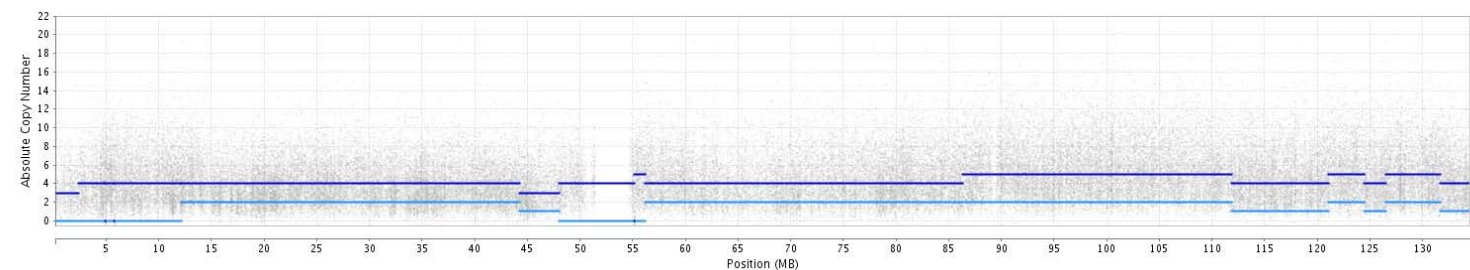


Figure 1. COSMIC Graphical Representations for Cell Lines

Figure 1: A graphical representation of copy number of chromosome 11 in cell lines T24, J82, SW1710, and VM-CuB-1 as presented by COSMIC (2014). The graphical representation of CNV in HTB9 cannot be shown as COSMIC (as of 2015) no longer gives the option to view the graph. On the bottom axis is the megabase position on chromosome 11. The lines in the graph represent major and minor copy number. Based on the UCSC genome browser, the *ATM* region is exactly at 107 megabases. In the above example, T24 would be classified as copy number neutral as the major allele line is at 2 copies.

Table 1. Results of Database Mining

Cell Line	<i>ATM</i>	<i>CHEK1</i>	<i>MRE11A</i>	<i>H2AFX</i>	<i>TP53</i>
HTB9	Normal	WT-E / Normal	Gain	Normal	MUT*
J82	Loss	Loss	Gain	Loss	MUT
SW1710	Loss	Loss	Loss	Loss	MUT*
T24	Gain	Normal/Gain	Gain	Normal/Gain	MUT
VM-CuB-1	Loss	Loss	Normal/Gain	Loss	MUT*

* TP53 status is labeled as homozygous mutated, deleted, etc. according to IARC TP53.

3.2 QUALITY ASSURANCE OF THE CELL LINES

A complete STR profile for each cell line was obtained at the beginning and the end of the project. This was to ensure that the cell lines were not entirely altered or been switched. The STR profile was determined and verified using the program called GeneMapper Version 4.0 from Applied Biosystems. According to the STR profiler provided online by DSMZ, all of the cell lines used match the database with greater than 90% accuracy. Table 2 represents the results determined from the STR profile at the completion of our study.

Table 2. STR Profiling Results

Sample	<i>Amelogenin</i>	<i>CSFIPO</i>	D13S317	D16S539	D5S818	D7S820	<i>THO1</i>	<i>TPOX</i>	VWA
HTB9	X,Y	11	11	9	11,12	10,11	7,9	8,9	16,18
J82	X,Y	10,11	10,12	11,12	8,12	9,11	9.3	11,12	17,18
SW1710	X	11,12	12	8,11	8,12	8,11	7,9.3	9,11	16,17
T24	X	10,12	12	9	8,10,12	9,10,11	6	8,11	17
VM- CuB-1	X,Y	11	10	11,12	8,11	8,11	9	8	18,19

Sample	<i>Amelogenin</i>	<i>CSFIPO</i>	D13S317	D16S539	D5S818	D7S820	<i>THO1</i>	<i>TPOX</i>	VWA
HTB9	X	11	11	9	11,12	10,11	7,9.3	10,11	18
J82	X,Y	10,11	10,12	11,12	12,13	9,11	9.3	11,12	17,18
SW1710	X	11,12	12,12	8,11	12	8,11	7,9.3	9,11	16,17
T24	X	10,12	12	9	10	9,11	6	8,11	17
VM- CuB-1	X	11	10	11,12	11	8	9	8	18,19

3.3 FLUORESCENCE IN SITU HYBRIDIZATION FOR *ATM* RESULTS

Bladder cancer cell lines HTB9, J82, T24, SW1710, and VM-CuB-1 were used in this study based on their copy number status of *ATM* as presented in the Catalogue of Somatic Mutations in Cancer (2013). Dual-color FISH with an *ATM* deletion probe that detects the *ATM* gene and a probe for the centromere of chromosome 11 was used to verify the *ATM* copy number. Copy number was determined using the ratio of the *ATM* gene signal (red) to the chromosome 11 centromere signal (green).

Copy number loss of *ATM* was seen in bladder cancer cell lines HTB9, J82, SW1710, and VM-CuB-1. Cell line VM-CuB-1 had *ATM* copy number loss in 79.1% of the cells. HTB9 had copy number loss in 78.2% of cells. Cell lines J82 and SW1710 had the highest percentage of copy number loss at 92.3% and 93.1%, respectively. T24 was classified as copy number neutral/gain for *ATM* because the FISH results show that 96% of the cells from this cell line had copy number gain or neutral copy number for *ATM*. Images that are representative of *ATM* and centromere 11 copy number of each cell line are presented in Figure 2. The summary of the FISH results is shown in Table 3.

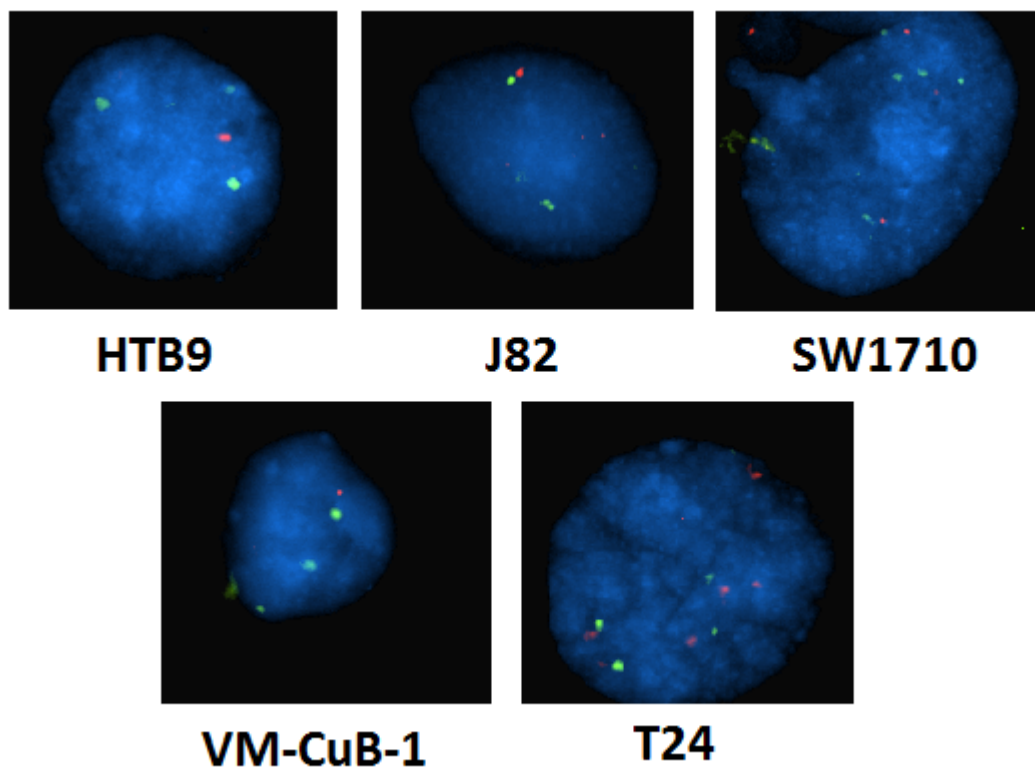


Figure 2. Fluorescence in situ Hybridization Images

Figure 2: Fluorescence in situ hybridization images using an *ATM* deletion probe set for each cell line. Green represents the centromere of chromosome 11, while red represents the *ATM* gene.

Table 3. Fluorescence in situ Hybridization Results

Cell Line	Gain/neutral	Loss	# of Cells Counted	Percentage Loss	Percentage Gain/neutral	Copy Number Status
VM-CuB-1	51	193	244	79.1	20.9	Loss
J82	19	228	247	92.3	7.7	Loss
HTB9	49	176	225	78.2	21.8	Loss
T24	197	8	205	3.9	96.1	Gain
SW1710	15	201	216	93.1	6.9	Loss

3.4 TP53 WESTERN BLOTTING RESULTS

TP53 is phosphorylated by ATM to arrest the cell in the G1 phase of the cell cycle. Defective TP53 function is a component of the radioresistance biomarker in OSCC (Sankunny et al., 2014). Therefore, it is critical to determine the functionality of TP53. Thus, we carried out a western blot to assess TP53 signalling to CDKN1A (p21) on proteins extracted from the bladder cancer cell lines.

TP53 expression can be seen in all cell lines. However, the protein structure of TP53 may be altered in some of the cell lines as the bands are higher or lower than what is expected in these cell lines. p21 is expressed in all cell lines, except T24. Based on the p21 expression, we

conclude that T24 has defective, non-functional TP53, while all other cell lines have a functional TP53 (Figures 3-5).

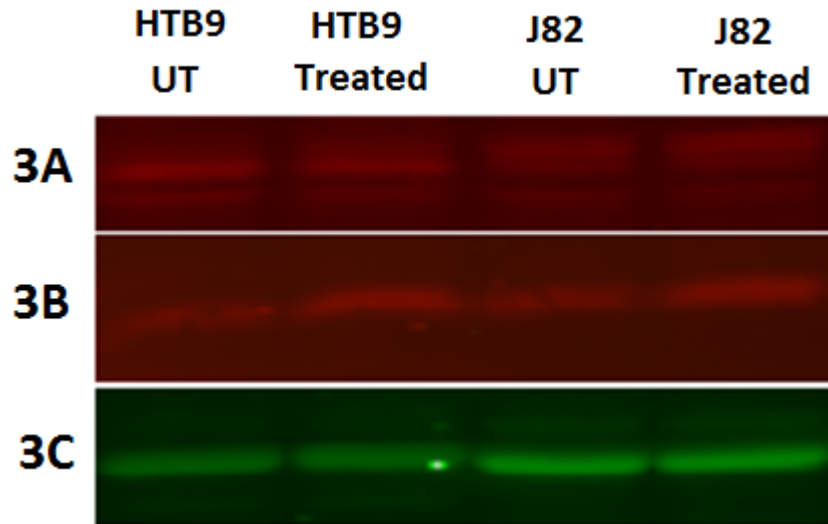


Figure 3. Western Blotting for HTB9 and J82

Figure 3: Western blotting illustrating the expression of TP53 (3A), p21 (3B), and alpha-beta tubulin (3C), the latter as a loading control for cell lines HTB9 and J82. From left to right, lanes represent HTB9 untreated, HTB9 doxorubicin-treated, J82 untreated, and J82 doxorubicin-treated.

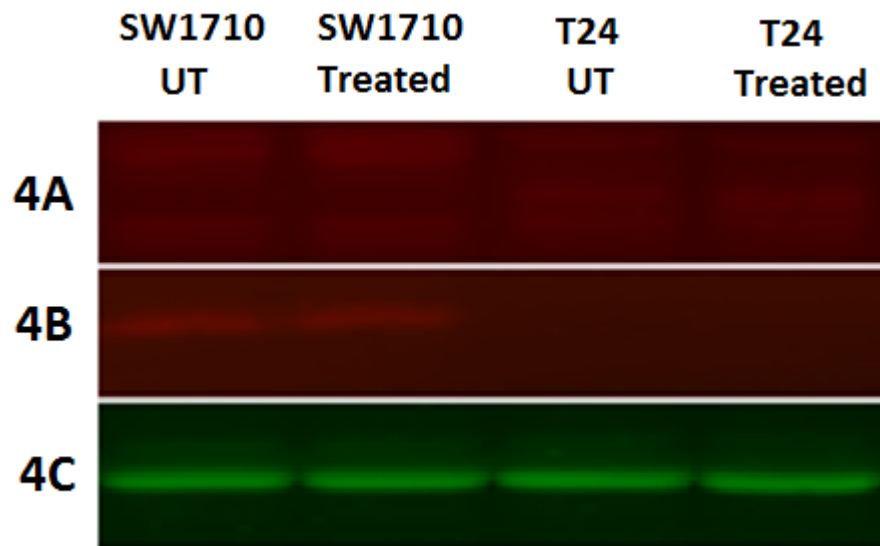


Figure 4. Western Blotting for SW1710 and T24

Figure 4: Western blotting illustrating the expression of TP53 (4A), TP21 (4B), and alpha-beta tubulin (4C), the latter as a loading control for cell lines SW1710 and T24. From left to right, lanes represent SW1710 untreated, SW1710 doxorubicin-treated, T24 untreated, and T24 doxorubicin-treated.

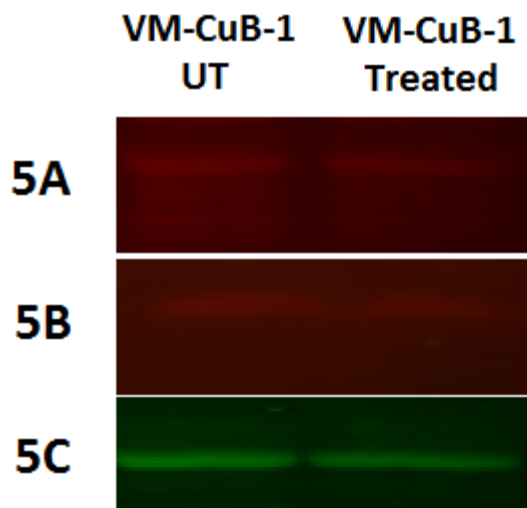


Figure 5. Western Blotting for VM-CuB-1

Figure 5: Western blotting illustrating the expression of TP53 (5A), TP21 (5B), and alpha-beta tubulin (5C), the latter as a loading control for cell line VM-CuB-1. From left to right, the lanes represent VM-CuB-1 untreated and VM-CuB-1 doxorubicin-treated.

3.5 RADIATION SENSITIVITY RESULTS

Clonogenic survival assays were used to determine if a cell line was radioresistant or radiosensitive. The assays were performed in triplicate and the surviving fractions for each subset of treatments (0 Gy, 2.5 Gy, or 5 Gy) were averaged together. The plating efficiency was determined based on the untreated controls using the following formula:

$$(\# \text{ of Cells Counted})/(\# \text{ of Cells Seeded})$$

The surviving fraction was determined using the following formula:

$$(\# \text{ of Cells Counted})/(\# \text{ of Cells Seeded} * (\text{Plating Efficiency}/100))$$

Overall, general survival of bladder cancer cell lines appears to be between 18% and 71% at 2.5 Gy and 1% and 15% at 5 Gy. The cell line with the lowest surviving fraction is HTB9 at

19% at 2.5 Gy and 1.2% at 5 Gy. The surviving fractions at either level of irradiation between cell lines T24, SW1710, and J82 were not statistically different. This would appear to disprove our initial hypothesis as the cell line with the lowest level of survival has *ATM* copy number loss. Figure 6 shows the average surviving fraction of HTB9, J82, T24, and SW1710 as performed by Dale W. Lewis from the University of Pittsburgh Cell Culture and Cytogenetics Core Facility.

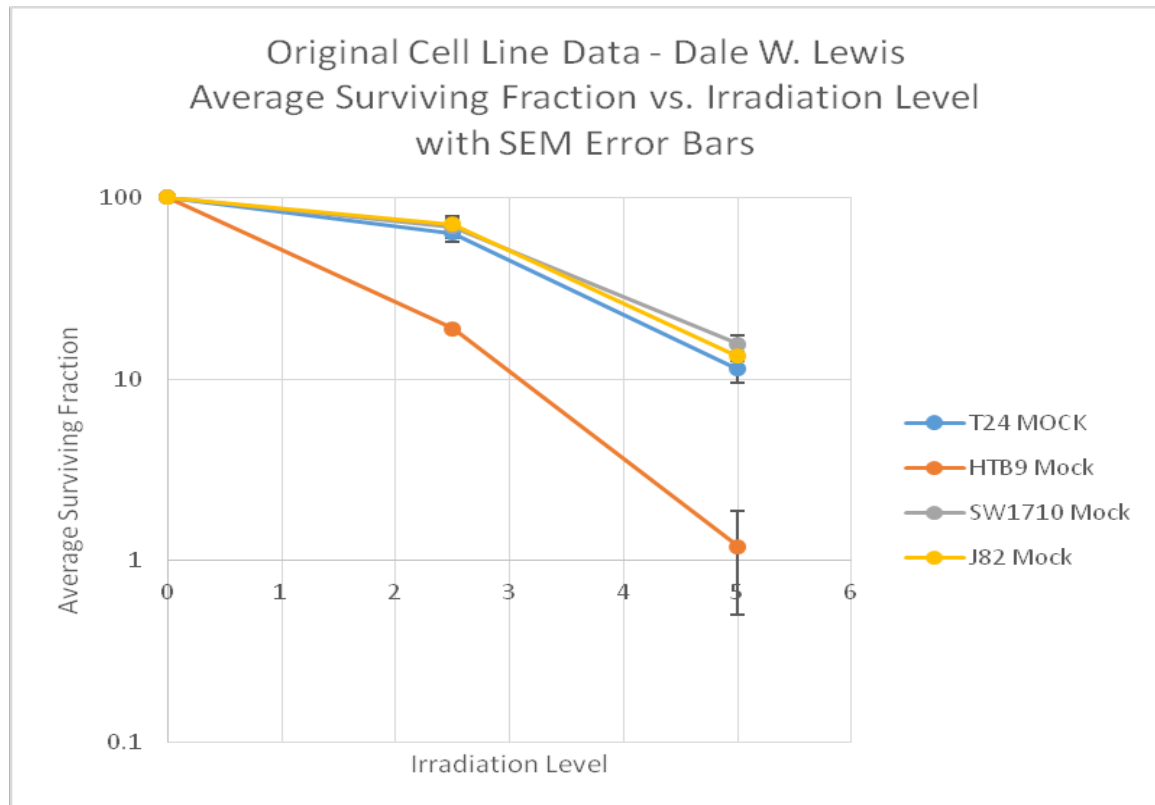


Figure 6. Clonogenic Survival Assay – Dale Lewis

Figure 6: The average surviving fraction compared to irradiation level for cell lines T24, HTB9, SW1710, and J82 as performed by Dale W. Lewis. The graph shows that HTB9 (a cell line with loss of *ATM*) has the lowest surviving fraction while all other tested cell lines have no statistical difference.

In order to validate the previous results presented by Dale W. Lewis, additional clonogenic survival assays were carried out. The total number of new clonogenic survival assays carried out for each cell line were: three for T24, two for SW1710, J82, and HTB9, and two for

VM-CuB-1. The extra clonogenic survival assay for T24 was carried out because it is the only cell line with copy number neutral/gain for *ATM*. The surviving fractions for 2.5 Gy of radiation ranged between 13.4 to 70.7% for T24, 6.9% to 23.1% for HTB9, 23.8 to 87.5% for SW1710, 17.4 to 80.1% for J82, and 31.8 to 38.7% for VM-CuB-1. At 5 Gy of radiation, the surviving fractions were 0 to 28.6% for T24, 0 to 2.55% for HTB9, 0 to 19.3% for SW1710, 0.6 to 21.2% for J82, and 5.3 to 11.05% for VM-CuB-1 (Figures 7-11) Figure 12 shows the mean of all of the surviving fractions across all of the clonogenic experiments. Inspection of these surviving fractions reveals that the cell line with the lowest surviving fraction is HTB9 (a cell line with loss of *ATM* and functional TP53). All other cell lines have similar mean surviving fractions despite *ATM* copy number loss and TP53 functionality. A summary of all results can be seen in Table 4.

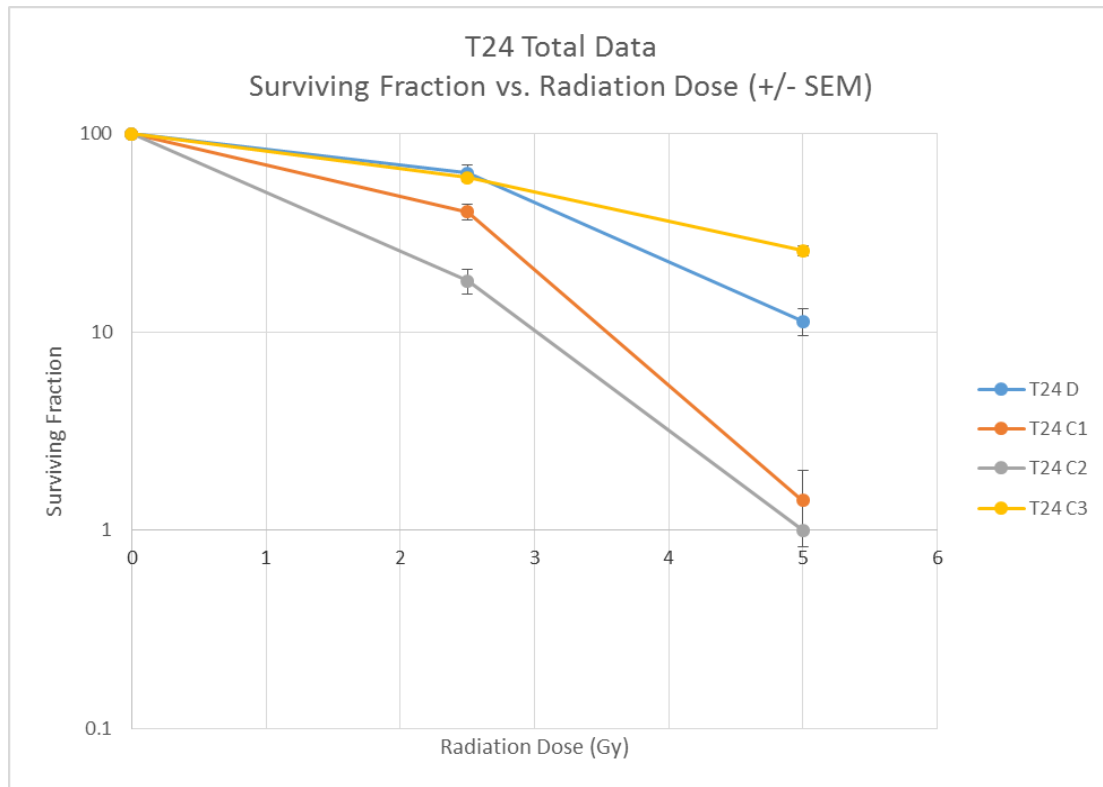


Figure 7. Clonogenic Survival Assay – T24

Figure 7: The mean surviving fraction for bladder cancer cell line T24 plotted against radiation dose (Gy). Each point is indicative of the mean (\pm SEM) of survival assays carried out in triplicate. Lines denoted with a “D” represents the data from Dale W. Lewis, while lines denoted with a “C” are data carried out by Chad H. Lawrence. The y-axis is a logarithmic scale. For T24 C2, the surviving fraction at 5 Gy is actually 0 rather than 1 (as 0 can not be plotted on a logarithmic scale).

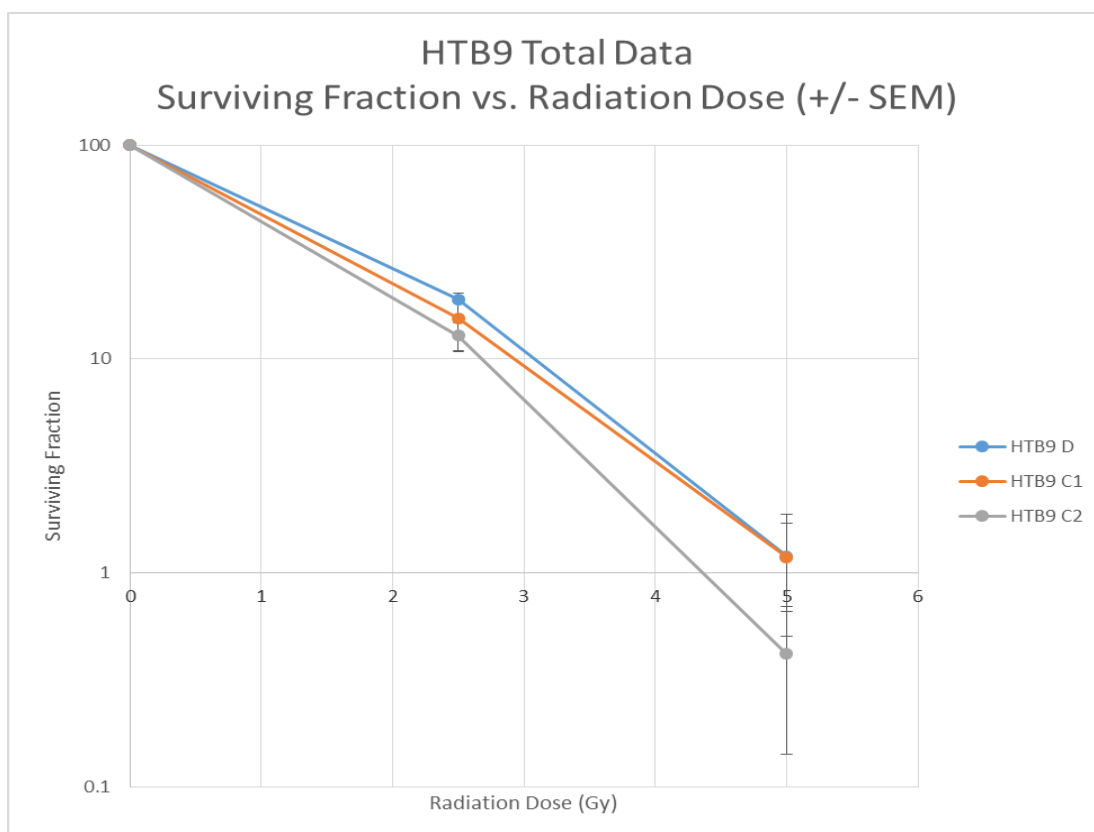


Figure 8. Clogenic Survival Assay HTB9

Figure 8: The mean surviving fraction for bladder cancer cell line HTB9 plotted against radiation dose (Gy). Each point is indicative of the mean (+/- SEM) of survival assays carried out in triplicate. Lines denoted with a “D” represents the data from Dale W. Lewis, while lines denoted with a “C” are data carried out by Chad H. Lawrence. The y-axis is a logarithmic scale.

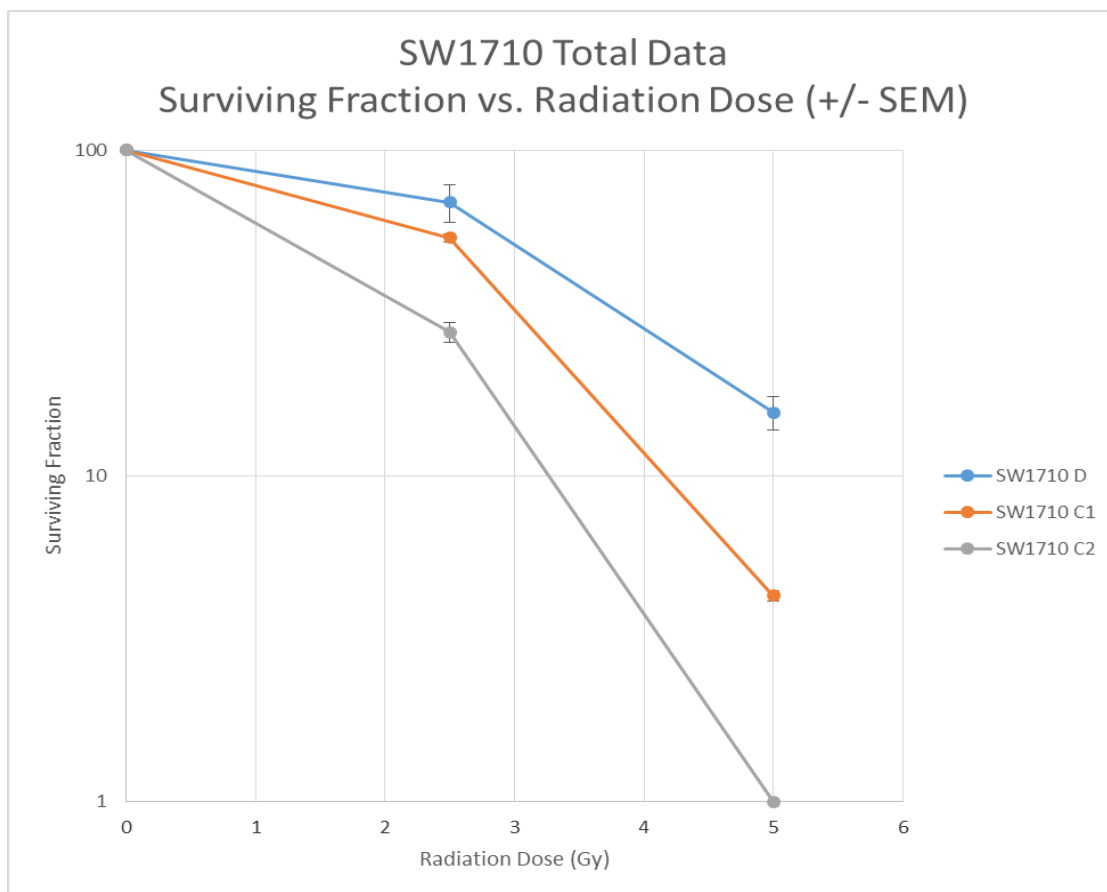


Figure 9. Clonogenic Survival Assay - SW1710

Figure 9: The mean surviving fraction for bladder cancer cell line SW1710 plotted against radiation dose (Gy). Each point is indicative of the mean (\pm SEM) of survival assays carried out in triplicate. Lines denoted with a “D” represents the data from Dale W. Lewis, while lines denoted with a “C” are data carried out by Chad H. Lawrence. The y-axis is a logarithmic scale. For SW1710 C2, the surviving fraction at 5 Gy is actually 0 rather than 1 (as 0 can not be plotted on a logarithmic scale).

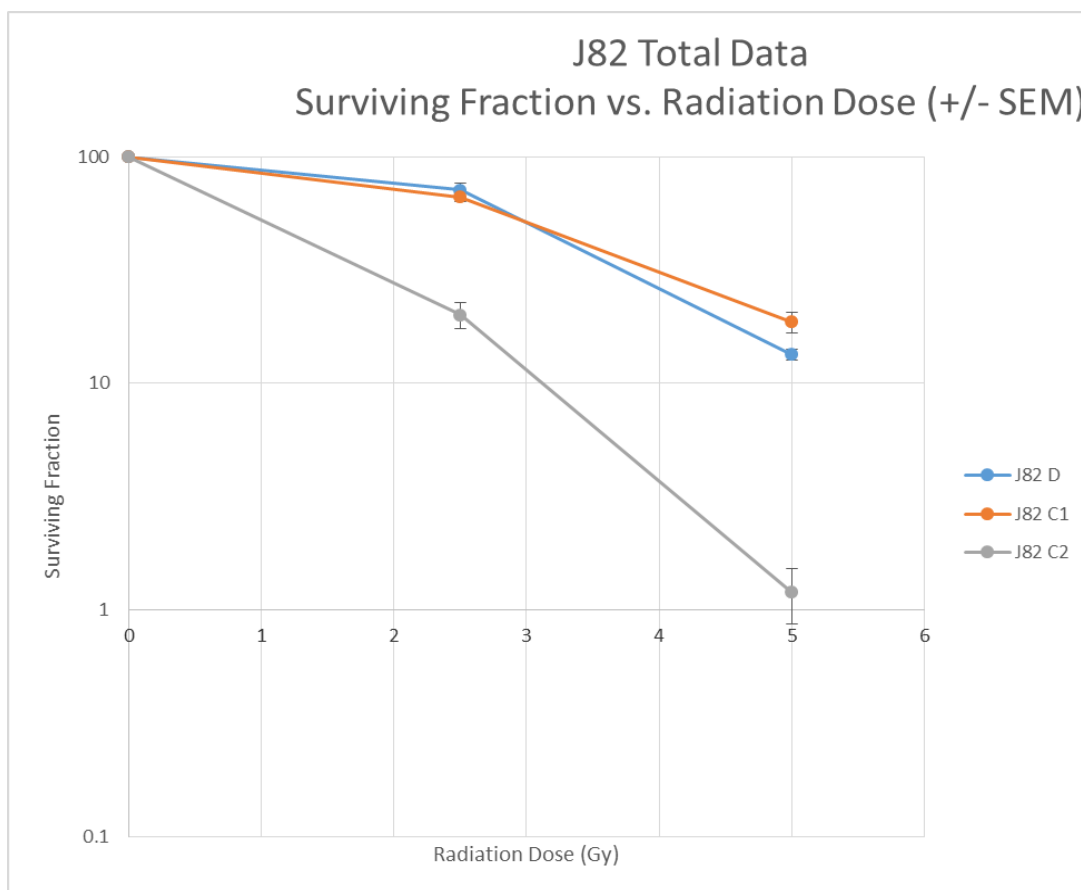


Figure 10. Clongenic Survival Assay - J82

Figure 10: The mean surviving fraction for bladder cancer cell line J82 plotted against radiation dose (Gy). Each point is indicative of the mean (+/- SEM) of survival assays carried out in triplicate. Lines denoted with a “D” represents the data from Dale W. Lewis, while lines denoted with a “C” are data carried out by Chad H. Lawrence. The y-axis is a logarithmic scale.

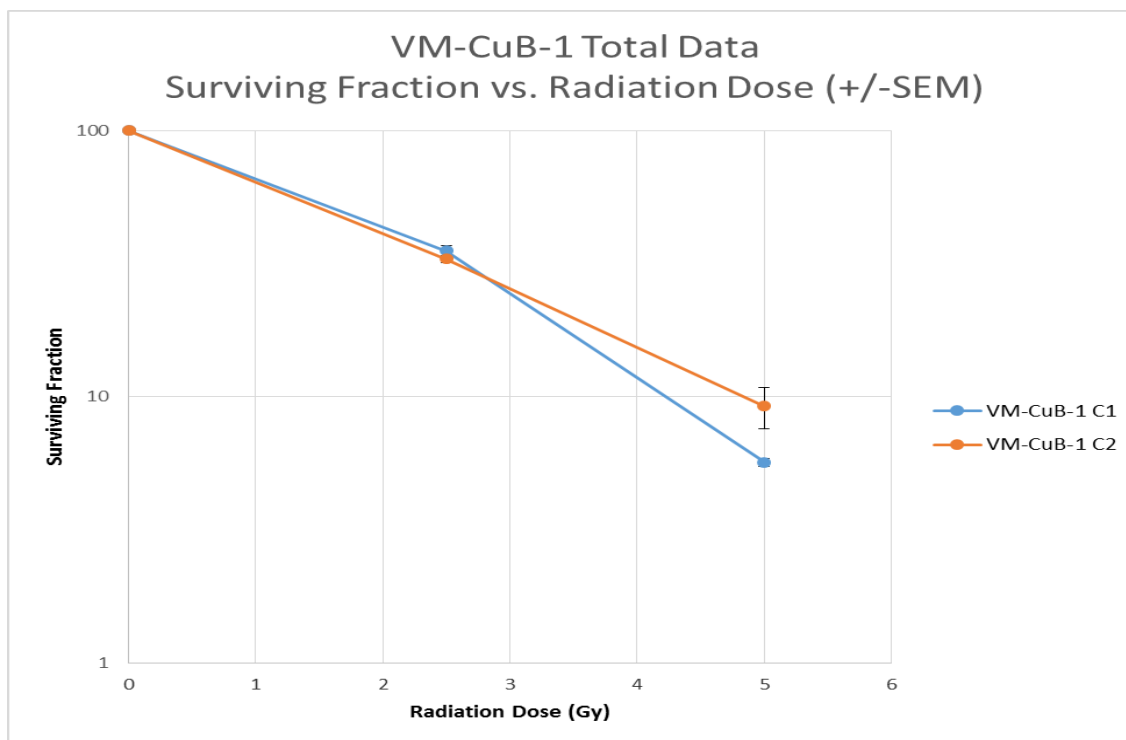


Figure 11. Clongenic Survival Assay - VM-CuB-1

Figure 11: The mean surviving fraction for bladder cancer cell line VM-CuB-1 plotted against radiation dose (Gy). Each point is indicative of the mean (+/- SEM) of survival assays carried out in triplicate. Lines denoted with a “D” represents the data from Dale W. Lewis, while lines denoted with a “C” are data carried out by Chad H. Lawrence. The y-axis is a logarithmic scale.

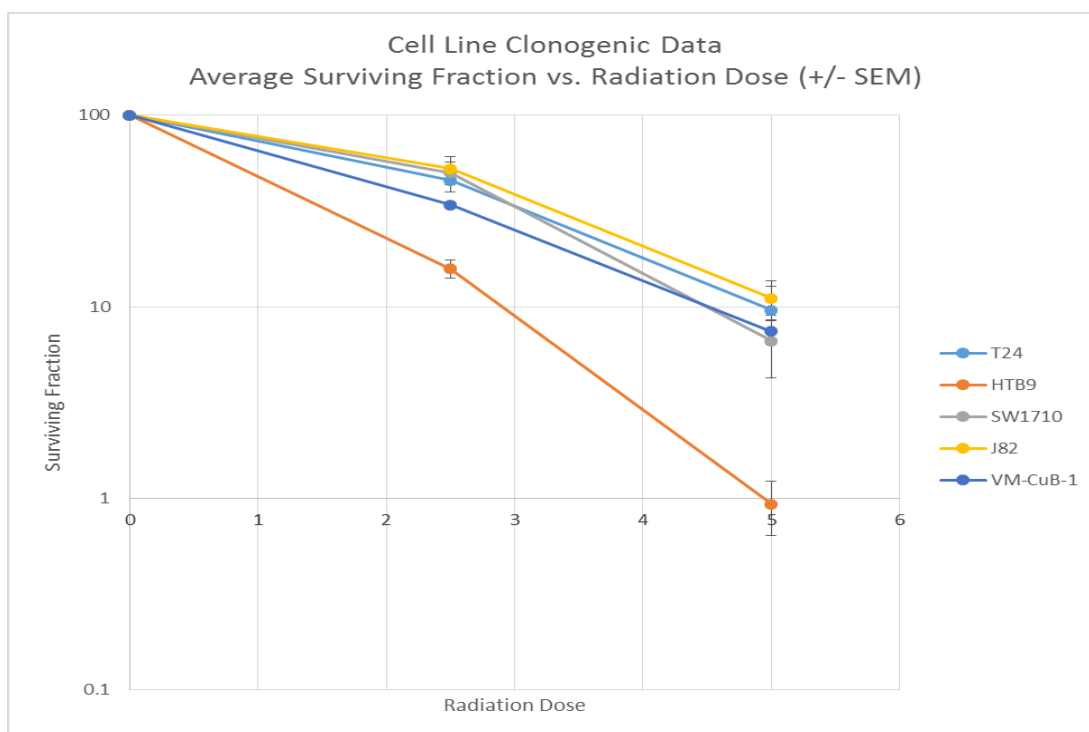


Figure 12. Clonogenic Survival Assay Summary

Figure 12: The average surviving fraction (+/- SEM) for cell lines T24 (N=12), SW1710 (N=9), HTB9 (N=9), J82 (N=9), and VM-CuB-1 (N=6) plotted against radiation dose. The y-axis is a logarithmic scale.

Table 4. Summary of Results

Sample Name	TP53 Status	<i>ATM</i> Copy Number	IR Status
HTB9	Functional	Loss	Sensitive
J82	Functional	Loss	Resistant
SW1710	Functional	Loss	Resistant
T24	Non-functional	Gain/neutral	Resistant
VM-CuB-1	Functional	Loss	Resistant

4.0 DISCUSSION

Cancer is a public health problem in the global community. Radioresistant cancer cell lines have been seen in previous studies, where it was hypothesized that the copy number loss of the *ATM* gene combined with a defective TP53 pathway may be used as a biomarker to predict radioresistance in head and neck squamous cell carcinomas. The purpose of this study was to determine if either copy number loss of *ATM* or the functionality of TP53 would explain the radioresistance observed in cancer cells.

The FISH data show that copy number loss of *ATM* is a feature of the bladder cancer cell lines HTB9, J82, SW1710, and VM-CuB-1. Gain/neutral copy number is a characteristic of the cell line T24. According to the clonogenic data, the survival response to radiation is similar in all cell lines, except HTB9. If our hypothesis that copy number loss of *ATM* (or TP53 functionality) was the sole cause for radioresistance in urothelial bladder cancer cell lines, T24 should have been the most radiosensitive cell line. Since we do not see this result, *ATM* may not be an accurate biomarker of radioresistance in bladder cancer cell lines. Although *ATM* may not be a biomarker for radioresistance in bladder cancer cells, perhaps other genes located on distal 11q may be.

One of these genes may be miR100. miR100 is one of the microRNAs in the miR99 family (Muller et al, 2013). The miR99 family has been shown to be upregulated following IR induced DSB (Muller et al., 2013). miR100, in particular, has been linked with decreasing

cellular proliferation in cancer cells (Morais et al., 2014). Another study to confirm this decrease in cellular proliferation involved the cell line 5637, which is HTB9 according to ATCC (Oliveira et al., 2011). Upregulation of the miR99 family has been shown to be linked with radiosensitivity in cells (Muller et al., 2013). However, loss of miR100 has been hypothesized to contribute to radioresistance (Henson et al., 2009).

MicroRNAs, in general, have been shown to affect the expression levels of different genes (Muller et al., 2013). Therefore, upregulation of the miR99 family may decrease the expression of other genes. *SMARCA5* is one of the genes targeted by miR100 (Morais et al., 2014). miR100 appears to control the expression patterns of *SMARCA5* (Morais et al., 2014). In particular, when miR100 is upregulated, *SMARCA5* becomes downregulated (Morias et al., 2014). The decreased expression of *SMARCA5* can lead to radiosensitivity, as shown in siRNA knockdowns of *SMARCA5* (Mueller et al., 2013).

Recent studies have suggested that miR100 can play a role in regulating the expression of *ATM* itself (Ng et al, 2010). According to the study by Ng et al, the 3' UTR region of *ATM* has a binding site for miR100. These investigators showed that when miR100 is upregulated, *ATM* expression is downregulated (Ng et al, 2010). Based on these results, it is possible that radioresistant cells in cancers may result from copy number (or possibly complete) loss of miR100.

In light of these studies, perhaps a more suitable biomarker for radioresistance may be miR100 instead of *ATM*. Future experiments should examine the copy number of miR100 and its expression. The copy number and expression of *SMARCA5* should also be examined. Clonogenic survival assays should be performed to determine whether these genes are useful candidates of radioresistance.

As with every experiment, errors may arise that can affect the outcome/results of the experiment. This study is no exception. In the clonogenic survival assays, the individual cell lines behave differently across technical replicates. In setting up the clonogenic survival assays, it is a common practice to stack the culture dishes on top of one another in a lead cylinder and then expose them to IR. This could lead to some culture dishes receiving more IR than others. This could be one explanation as to the vastly different surviving fractions, as in one replicate the culture dish may be exposed to higher IR (thus yielding lower SF), while the other replicate is exposed to lower IR (thus yielding a higher SF). This, however, may not explain all of the fluctuations we see amongst the SF. In counting the colonies that survive IR, it is left to the observer to make the “call” if the colony has grown to greater than 50 cells. Sometimes, cells can grow in three-dimensional space. However, the clonogenic survival assays are observed in a nearly two-dimensional space. Therefore, some colonies that exhibit this three dimensional growth may be erroneously classified as less than 50 surviving cells. However, this is unlikely to cause such wild fluctuations as three-dimensional growth in non-confluent cell lines is rather rare. Another factor that could contribute to these inconsistencies is the fact that we are working with heterogeneous cell lines (in the sense that there are some cells with copy number loss of *ATM* while other cells in the same cell line exhibit copy number gain). This could be a contributing factor because one culture dish may get more cells with copy number gain than the other culture dishes, even if they are plated at the same time. This could yield some assays with higher survival than others when comparing assays from the same cell line. Lastly, we are assuming that when we plate the cells we have a single cell suspension. While we take extra steps to try to minimize this source of error, sometimes it can be unavoidable. This can contribute to the fluctuations seen in the clonogenic assays as it would lower the number of

potential colonies that could form. The lower potential will be displayed in the surviving fractions as we assume each of the 2000 cells has the ability to form its own colony (something that cannot be achieved if the cells are clustered, even in pairs of two).

In conclusion, determining the exact behavior of cancer cells is challenging. Our clonogenic survival assay results suggest that copy number loss of *ATM* (with no other factors considered) may not be the best biomarker for predicting radioresistance/radiosensitivity in urothelial bladder cancer cell lines. Other studies using treatment of cancer cells with ionizing radiation have suggested other biomarkers, such as miR100 and *SMARCA5*. It is quite possible that radioresistance is based on a combination of factors such as copy number of *ATM*, TP53 functionality, copy number and/or expression of *SMARCA5*, and the copy number and/or expression of miR100. Future studies should examine each of these conditions, or any possible combinations, in order to determine which of these would be the most suitable biomarker for radioresistance bladder cancer cell lines.

APPENDIX A: ANTIBODIES USED FOR WESTERN BLOTTING

Antibody	Type	Company	Concentration
Total p53 (M-19)	Goat Polyclonal	Santa Cruz Biotechnology	1 - 1000
Total p21 (SC-817)	Mouse Monoclonal	Santa Cruz Biotechnology	1 - 1000
Alpha-Beta Tubulin	Rabbit Polyclonal	Cell Signaling	1 -1000
Anti-Mouse (Secondary)	IRDye 680LT Goat Anti-Mouse	LiCOR	1 - 10,000
Anti-Goat (Secondary)	IRDye 680LT Donkey Anti-Goat	LiCOR	1 - 10,000
Anti-Rabbit (Secondary)	IRDye 800CW Donkey Anti-Rabbit	LiCOR	1 - 10,000

APPENDIX B: DETAILED PROTOCOLS USED IN THE PROJECT

B.1 Cell Culture Protocol

Medium Preparation

1. Prewarm the appropriate medium (McCoy's 5A, DMEM, EMEM, or RPMI 1640) and 55 mL Fetal Bovine Serum (FBS) in a waterbath set at 37 degrees Celsius for at least 20 minutes or until thawed. Information as to which medium the cell line requires can be found at DSMZ or ATCC.
2. Prewarm 5 mL of L-Glutamine solution in a waterbath set at 37 degrees Celsius until thawed.
3. Wipe down all bench tops (including the inside surface of the laminar flow biosafety cabinet) and instruments down with 70% ethanol.
4. Wipe all bottles and reagent tubes down with 70% ethanol before placing in a laminar flow biosafety cabinet.
5. To 500 mL of medium, add 55 mL FBS (final concentration of 10%) , 5 mL L-Glutamine (1% final concentration) and 5.5 mL non-essential amino acids (NEAA) (1% final concentration).
6. Remove 100 mL of this mixture and filter the solution using a 60 cc Syringe and a 0.22 micron filter (60 mL once, then 40 mL). Store this in a -4°C refrigerator. This will be used for any cell culture requiring antibiotic free media.
7. To the remaining medium, add 450 uL of Gentamycin (final concentration of 0.05 mg/mL). This will be referred to as "supplemented medium" throughout the protocols.
8. Pass the solution through a steriflip filter (0.22 microns).
9. Prepare a solution consisting of 10% Dimethyl Sulfoxide (DMSO) in supplemented medium by adding 5mL of DMSO to 45 mL of supplemented medium. This will be used in Cryopreservation of Cell Lines.
10. Filter the contents using a 60cc Syringe and a 0.22 micron filter. Shield the tube from light using aluminum foil.
11. Store all media in a -4°C refrigerator.

Thawing the Cell Lines and setting up a Mycoplasma Test

1. Prewarm the antibiotic free medium as well as the medium supplemented with FBS, NEAA, L-Glutamine, and gentamicin in a waterbath set at 37°C for at least 20 minutes.
2. Obtain the cryopreserved cell culture from a nitrogen freezer.
3. Immediately place the cryotube into a waterbath set at 37°C.
4. Wipe down all Bench tops, including the laminar flow biosafety cabinet, with 70% Ethanol.
5. Wipe all bottles and tubes down with 70% ethanol before placing anything in the laminar flow biosafety cabinet.
6. Using a Sterile Serological Pipette, draw up 4 mL of supplemented medium and then the contents of the Cryotube.
7. Pipette the mixture into a sterile 15 mL conical Polystyrene tube.
8. Centrifuge the tube at 438 xG for 8 minutes at 22°C.
9. While the tube is spinning, add a sterile 22x22 mm coverslip to a sterile 35mm Culture dish. Label this culture dish with the name of the cell line, "Mycoplasma Test", the date, and your initials.
10. After the tube is finished spinning, move it into the hood and aspirate the excess media using a sterile 9" Pasture pipette and the vacuum in the biosafety hood into a 500 mL capacity Vacuum Flask, leaving only the pellet of cells behind.
11. Resuspend the pellet in 5 mL of Supplemented Media by either tapping the side of the tube or pipetting up and down using a sterile 5 mL serological pipette.
12. Add a few drops of this cell suspension to the 22x22 mm coverslip.
13. Add the rest of the cell suspension to a Corning 25mm flask (T25). Label the T25 flask with the cell line, passage number (if applicable), date, the medium used, and your initials.
14. Add 1mL of antibiotic free medium onto the coverslip, being careful not to have the medium spill into the surrounding culture dish.
15. Place the T25 flask and the culture dish into an incubator set at 37°C / 5% CO₂
16. NOTE: Cell lines should be evaluated using an inverted phase contrast microscope and split or fed every 2-3 days.

Mycoplasma Testing

1. Two to three days after setting up the culture dish (described in the previous section), prepare Carnoy's Fixative by combining acetone-free Methanol : Glacial Acetic Acid in a ratio of 3:1.
2. Remove the culture dish from the incubator and move it into a chemical hood.
3. Add 5-10 drops of Carnoy's Fixative to the coverslip. Let stand for 5 minutes.

4. Drain the coverslip into an empty biohazard collection bottle or an existing Biohazard Collection Bottle and add 5-10 more drops of Carnoy's Fixative to the coverslip. Let stand for 5 minutes. If using a new Collection Bottle, place an orange Chemical Hazard Label on the bottle and mark the chemical hazard in the bottle, the quantity of the chemical, the department, and sign and date the label.
5. Carefully drain the coverslip of fixative into the Biohazard Collection Bottle and allow to air dry.
6. Add 1mL of DAPI (4',6-diamidino-2-phenylindole) to the coverslip. Let stand for 2 minutes while protecting the coverslip from direct light.
7. Wash the coverslip with deionized water to remove the residual DAPI. Let the coverslip air dry.
8. Add 10-20 uL of "antifade" to a glass slide.
9. Mount the coverslip in the antifade onto the slide so that the cells are facing the slide.
10. Assess the nuclei of the stained cells for mycoplasma (see NOTE). If the culture is mycoplasma-positive, remove the T25 flask from the incubator immediately and sterilize the incubator.

NOTE: A positive Mycoplasma test is defined as seeing blue speckled particles around the nucleus of a cell or in the surrounding areas.

Feeding the Cell Line

1. Prewarm all media in a waterbath set at 37 degrees Celsius for at least 20 minutes.
2. Wipe of all surfaces, instruments, bottles, and tubes with 70% Ethanol before use
3. Using an inverted phase contrast microscope, assess the confluency of the cell lines in the T25 flask. If the confluency is <70%, perform the following steps. If the confluency is >70%, move to "Splitting the Cell Line" section.
4. Move the T25 flask into a biosafety hood.
5. Carefully aspirate the media from the flask using a 9" sterile Pasteur pipette, a vacuum, and a 500 mL capacity Vacuum Flask, ensuring that the pipette does not come into contact with the side of the flask that is growing the cells or any other surface.
6. Add 5mL of the appropriate supplemented medium to the T25 Flask.
7. Return the T25 Flask to the incubator.

Splitting the Cell Line

1. Prewarm all media, Hanks Balanced Salts Solution (HBSS), and 0.05% Trypsin-EDTA in a waterbath set at 37 degrees Celsius.
2. Wipe of all surfaces, instruments, bottles, and tubes with 70% ethanol before use
3. Move the T25 flask of the cell line with >70% confluency into a biosafety hood.
4. Aspirate the medium from the T25 flask.

5. Add 5 mL of HBSS to the flask. Let stand for 1-2 minutes.
6. Aspirate the HBSS from the flask and add 2-3 mL of 0.05% Trypsin-EDTA to the flask.
7. Return the flask to the incubator and let stand for 2-5 minutes (varying depending on cell line). This allows the Trypsin to react faster in the flask, thus detaching the cells from the surface.
8. Remove the flask from the incubator and gently tap the flask against a hard surface. Assess if the cells have detached using an inverse phase microscope.
9. If the cells have detached, move the flask into the Biohazard Safety Hood and add 8mL of Supplemented media. If they have not, return to step 7 (the time varies based on how many cells, if any, have detached).
10. Pipette 5mL of the solution to a new T25 flask. Label this flask with the cell line's name, the medium that it is grown in, the date, and your initials. IF there is a passage number, be sure to mark that on the new flask as well. On the old flask, mark that the cell line was passaged as well as the ratio of the split cells (ie 1/3, 1/2, etc).
11. Return all flasks to the incubator.

Cryopreserving the Cell Line

1. Prewarm the appropriate supplemented medium, HBSS, 0.05% Trypsin, and 10% DMSO solution in a 37°C waterbath for at least 20 minutes.
2. Wipe of all surfaces, instruments, bottles, and tubes with 70% ethanol before use.
3. Move the T25 flask of the cell line to be cryopreserved to the biosafety hood.
4. Aspirate the media from the T25 flask using a sterile 9" Pasteur pipette, a vacuum, and a 500mL capacity vacuum flask.
5. Add 5mL of HBSS to the flask. Let stand for 1-2 minutes.
6. Aspirate the HBSS from the flask and add 2-3 mL of 0.05% Trypsin to the flask using a sterile 9" Pasteur pipette, a vacuum, and a 500mL capacity vacuum flask.
7. Return the flask to the incubator and let stand for 2-5 minutes (varying depending on cell line).
8. Remove the flask from the incubator and gently tap the flask against a hard surface. Assess if the cells have detached using an inverse phase microscope.
9. If the cells have detached, move the flask into the biosafety hood and add 2mL of Supplemented media. If they have not, return to step 12 (the time varies based on how many cells, if any, have detached).
10. Using a 5mL sterile Serological Pipette, move the mixture into a 15 mL tube.
11. Centrifuge the tube at 438 xG for 8 minutes at 22°C.
12. Aspirate the supernatant from the tube using a sterile 9" Pasteur pipette, a vacuum, and a 500mL capacity Vacuum flask.
13. Resuspend the pellet in 1mL of the 10% DMSO solution.
14. Move resuspended solution to a Cryotube. Label the tube with the cell line name, passage number (if available), the type of medium it is grown in, date, and your initials
15. Place the Cryotube in a -20 °C freezer.
16. After 24 hours, move the Cryotube to a -80 °C freezer.

17. After 24 hours, move the Cryotube to a nitrogen freezer. Make a note as to where the tube was placed and update any record book/software program to the location of the newly placed tube.

B.2 Fluorescence *in situ* Hybridization protocol

Harvesting the Cells

1. Assess the confluency of the cell line using an inverse phase microscope. If the confluency is greater than or equal to 70%, the following steps can be performed.
2. Prepare 0.075 M KCl hypotonic solution
3. Add 50 uL of Colcemid to the flask(s) containing the cell line that will be harvested. Allow this to sit in the 37°C incubator for 30-60 minutes.
4. Aspirate the media from the flask.
5. Add 5mL of Hypotonic Solution to the flask and carefully detach the cells from the side of the flask using a cell scraper.
6. Transfer the contents of the flask to a 15mL tube and place the tube in a 37 degree Celsius waterbath for 30 minutes.
7. While the tube is sitting in the waterbath, make Carnoy's fixative by adding 10 mL of Glacial Acetic Acid to 30 mL of Methanol.
8. After 30 minutes have elapsed, add 5 drops of Carnoy's fixative to the 15 mL tube. Let this stand for 5 minutes in the waterbath.
9. Centrifuge the tube for 8 minutes at 438 xG.
10. Aspirate the Supernatant.
11. Resuspend the pellet in 5 mL of Carnoy's fixative.
12. Centrifuge the tube for 8 minutes at 438 xG.
13. Aspirate the Supernatant.
14. Resuspend the pellet in 5 mL of Carnoy's fixative.
15. Centrifuge the tube for 8 minutes at 438 xG.
16. Aspirate the Supernatant.
17. Resuspend the pellet in 5 mL of Carnoy's fixative.
18. Centrifuge the tube for 8 minutes at 438 xG.
19. Aspirate the Supernatant.
20. Resuspend the pellet **in 2 mL** of Carnoy's fixative.
21. Prepare a glass slide by applying just enough Carnoy's fixative to coat the slide.
22. Take a glass pipette and remove some of the cell suspension from the 15 mL tube.
23. Add 3-4 drops of this cell suspension onto the glass slide (with about 3 feet distance from the slide and the glass pipette).
24. Gently Huff of the slide. DO NOT BLOW on the slide.
25. Place the slide on a slide warmer until the fixative has evaporated.
26. Assess the slide for cells in metaphase.

27. Mark a 22mm area (that contains the highest number of metaphase cells) by scratching the back of the slide with a diamond-tipped pen.
28. Store the slide in a -20 °C freezer until FISH can be performed.

Fluorescence in situ Hybridization (Day 1)

1. Mix 800 mL of water with 175.3 g of sodium chloride and 88.2 g of sodium citrate. pH this solution to 7.0. Add enough water to bring the volume to 1 L. This is 20x SSC. Store this at room temperature.
2. Prepare 70% formamide by mixing 35mL of formamide with 10 mL of Millipore water and 5 mL of 20x SSC. pH this solution to 7.0 and store in a 4 °C fridge until use.
3. Mix 100 mL of 20x SSC with 900 mL of Millipore water to create 2x SSC.
4. Set 2 waterbaths to 37 and 75 °C.
5. Place 47.5 mL of 2x SSC in the 37 °C waterbath.
6. Place the 70% formamide Coplin jar in the 75°C waterbath. NOTE: Do not wait for the waterbath to heat up to full temperature before adding the Coplin jar, as this will cause the jar to crack.
7. Add 2.5 mL of RNase to the 2x SSC at 37 °C and mix thoroughly.
8. Obtain the slide(s) to be used and place them in the 2x SSC/ RNase solution for 30 minutes.
9. Briefly vortex and centrifuge the probe (ATM deletion probe) and place in a shaker set at 37 °C for at least 15 minutes.
10. After 15 minutes, remove the probe from the shaker and place it in the 75 °C waterbath for 5 minutes.
11. Float the probe in the 37 °C waterbath for 15 minutes but not more than 30 minutes!
12. When the probe is set in the 37 °C waterbath, remove the slides from the RNase solution and immediately place in a Coplin jar containing 2x SSC for 2 minutes.
13. Remove the slides from the 2x SSC solution and place in the slides in a 70% ethanol solution at room temperature in a Coplin jar..
14. Remove the slides from the 70% ethanol solution and place in a 80% ethanol solution room temperature in a Coplin jar.
15. Remove the slides from the 80% ethanol solution and place in 100% ethanol room temperature in a Coplin jar.
16. Allow the slides to air dry on a slide warmer.
17. Once the slides are dry, place the slides in 70% formamide for 4 minutes.
18. Briefly dry the back of the slides off on a paper towel and place the slides in 70% ethanol for 2 minutes.
19. Place the slides in 80% ethanol for 1 minute, followed by 100% ethanol for another minute.
20. Dry the slides on the slide warmer. By now, anywhere between 15 minutes and 25 minutes should have elapsed since you put the probes in the 37 °C waterbath.

21. Remove the probes from the waterbath and pipette 10 uL of this onto the designated area of the slide.
22. Cover the slide with a 22x22 mm coverslip. Seal the edges of the coverslip with rubber cement and place the slides in a hybridization box.
23. Place the hybridization box into an oven set at 37 °C overnight.

FISH (Day 2)

1. Prepare Wash 1 by mixing 20 mL of 20x SSC with 950 mL of Millipore water and 3 mL of Tween 20. Adjust the pH to 7.0 and add enough water to bring the volume to 1L.
2. Prepare Wash 2 by mixing 100 mL of 20x SSC with 850 mL of water and 1 mL of Tween 20. Adjust the pH to 7.0 and bring the total volume to 1L using Millipore water.
3. Set a waterbath to 73 degrees Celsius.
4. Place Wash 1 into the waterbath. NOTE: do not wait until the waterbath has reached full temperature to add the jar.
5. Remove the slides from the hybridization box and carefully remove the rubber cement and coverslips.
6. Place the slides in the Wash 1 solution for 2 minutes.
7. Remove the slides and place them in Wash 2 solution for 2 minutes.
8. Place the slides in a jar containing 2x SSC at room temperature.
9. While in 2x SSC, obtain a jar of DAPI from a refrigerator.
10. Place the slides in the jar of cold DAPI for 2 minutes.
11. Remove the slides from the DAPI and place in a jar with Millipore water for 1 minute.
12. Place the slides in a jar containing 70% ethanol for 1 minute.
13. Place the slides in a jar containing 80% ethanol for 1 minute.
14. Remove the slides from the 80% ethanol and place them in a jar containing 100% ethanol for 1 minute at room temperature.
15. Allow the slides to air dry on a slide warmer.
16. Pipette 35 uL of antifade solution onto the slide.
17. Cover the slide with a 22x50 mm coverslip.
18. Carefully turn the slide upside down onto a paper towel and press gently.
19. Seal the edges of the coverslip with clear nail polish.
20. The slides can now be viewed under a Fluorescence microscope or stored in a slide box in a -20 degree freezer until they can be viewed.

B.3 Western Blotting Protocol

Preparing the Protein Lysate

1. Prepare Lysis Buffer by mixing 1 mL of 1M Tris, 15 mL of 0.5M NaCl, 5 mL of 0.5M EDTA, 5 mL of Triton x100, and 0.5 mL of 10% SDS. Bring the volume up to 500 mL using Millipore water.
2. Pipette 1mL of Lysis buffer to a 2mL tube and add 5uL Pepstatin, 4uL PMSF, and 40uL of Leupeptin. Keep on ice.

3. Remove the T25 flask (of the cell line whose protein is to be extracted) from the incubator and place in a biosafety hood.
4. Aspirate the media from the T25 flask.
5. Add 5mL of cold (4°C) 1x Phosphate Buffered Saline (PBS).
6. Using a Cell Scraper, remove the cells from the inside surface of the flask.
7. Pipette the PBS/cell suspension into a 15mL flask and centrifuge at 438 xG for 8 minutes.
8. Aspirate the supernatant from the tube and resuspend the cell in 100-200uL of Lysis Buffer supplemented with protease inhibitors. Amount of Lysis Buffer added is proportionally related to the size of the cell pellet.
9. Pipette the contents of the 15mL tube into a 2 mL microcentrifuge tube. Place the 2 mL tube on ice for 1 hour.
10. Centrifuge the 2mL tube at 13,557 xG for 30 minutes at 4°C.
11. Pipette the supernatant from the 2mL tube into a new 2 mL tube. Discard the old tube.
12. Keep the new tube in a -80°C freezer until further use.

Preparing the Acrylamide Gel

1. Prepare the resolving buffer by mixing 72.6 g of Tris Base in 350 mL of Millipore water. Adjust the pH to 8.8 using HCl. Add enough water to make a solution of 400 mLs.
2. Make the Stacking buffer by adding 6 g of Tris base to 100 mLs of water. Adjust the pH to 6.8.
3. Prepare 4x Laemmli buffer by mixing 2.5 mL of Tris stacking buffer, 2.5 mL of 10% SDS, 2.5 mL of Glycerol, 0.25 mL of Beta mercaptoethanol, and a very small amount of Bromophenol Blue. Adjust the pH to 7.6-7.8 and store at 4 degrees Celsius until use.
4. Obtain a 1.5mm inner glass plate and a glass outer plate. Wash these with 70% ethanol and deionized water.
5. Place the plates together in a green plate holder. Secure the clamps. Ensure that the edges of the glass plates are in line with one another.
6. To prepare a 12% Acrylamide gel, mix 5.1 mL of Millipore water, 3.75 mL of Resolving Buffer, 6.0 mL of 30:1 BiS Acrylamide, 150 uL of 10% SDS, 80 uL of APS, and 14 uL of TEMED into a 15mL tube. Quickly mix through inversion and pour in between the glass plates up to the top of the green holder (to allow room for the stacking gel).
7. Carefully pipette 1-2mL of Water-Saturated Butanol over the acrylamide gel in order to remove air bubbles.
8. Let stand for 1 hour or until acrylamide has solidified.
9. Carefully pour off the Water-saturated Butanol (or blot dry with wattman paper)
10. Rinse the gel with Deionized Water in order to remove any residual Butanol.
11. Prepare the stacking gel by mixing 3 mL of Millipore water, 1.25 mL of Stacking Buffer, 650 uL of 30:1 BiS Acrylamide, 50 uL of 10% SDS, 35 uL of APS, and 7 uL of TEMED into a 15mL tube.
12. Pour the stacking gel onto the resolving gel and immediately place a 1.5mm 15 Well comb into gel.

13. Allow the gel to sit for 30 Minutes – 1 Hour or until the gel has solidified in the tube.

Running Proteins through the Acrylamide Gel

1. Prepare SDS-Electrophoresis Running Buffer by mixing 3.05 g of Tris base, 14.4 g of Glycine, 1 g of SDS, and 1L of water.
2. Once the Gel has been cast, place it into the SDS-Electrophoresis Apparatus along with a Buffer Dam.
3. Pour the SDS-Electrophoresis Running Buffer in between the Gel and the Buffer Dam. Ensure there is no leaks.
4. Remove the 1.5mm Comb from the gel. Allow the Gel to equilibrate to the Buffer for at least 10 minutes.
5. Obtain the proteins to be run from the -80 Degree Freezer.
6. Float the tubes of protein in 100 degree Celsius water for 5 minutes.
7. Remove the tubes from the water, take 20uL of the protein solution and mix it with 5uL of Laemmli Buffer.
8. Pipette 20uL of the protein / Laemmli Buffer into one of the gel wells. Repeat this for every protein sample.
9. Add 2-5uL of Dual Color Protein Ladder (Biorad) to a separate gel well.
10. Fill the outside chamber of the apparatus with the SDS-Electrophoresis Running Buffer.
11. Place the electrodes to the apparatus, matching the color of the electrode to the spot on the apparatus.
12. Run the apparatus on 40-60 Volts for 1-2 hours or until adequate separation of the protein is achieved.

Transferring the Protein onto a Membrane

1. Cut 16 pieces of Wattman Paper to fit the cassettes of the Transfer Apparatus.
2. Cut a piece of Nitrocellulose membrane slightly smaller than the Wattman paper pieces.
3. Prepare the Transfer Solution by mixing 4.55 grams of Tris Base, 21.625 grams of Glycine, 800mL of Deionized Water, and 200mL of Methanol.
4. Soak the Wattman Pieces, the Nitrocellulose membrane, and the Cassette Sponges in the Transfer Buffer for 5 minutes.
5. Remove the Gel from the SDS-Electrophoresis apparatus and carefully remove the outer glass plate.
6. Cut away the Stacking Gel and any part of the Resolving Gel that does not have protein.
7. Open one of the Transfer Cassettes.
8. Add a sponge to the black side of the cassette.
9. Add 4 pieces of Wattman Paper to the sponge.
10. Place the Nitrocellulose over the Wattman Paper pieces.

11. Carefully place the Resolving Gel onto the Nitrocellulose membrane and pour some transfer buffer onto it.
12. Smooth out the Gel using the smooth side of a Serological pipette or a plastic roller. Ensure that there are no bubbles between the gel and the membrane.
13. Place 4 pieces of Wattman Paper over the gel and use a plastic roller to ensure that no bubbles formed between the gel and membrane in the process.
14. Cover the Wattman Paper with a Cassette Sponge.
15. Close the Cassette and place in the Transfer Apparatus (Matching black to black and white to red).
16. Place 8 Wattman paper pieces in between 2 Cassette Sponges and close the 2nd Transfer Cassette.
17. Place the Second Cassette in the Transfer Apparatus.
18. Place an ice container in the empty space of the Transfer Apparatus.
19. Fill the outside chamber of the Transfer Apparatus with Transfer Buffer.
20. Run the Transfer Apparatus at 80 Volts for 2 hours (replacing the ice container with a new one after the first hour). NOTE: Transfer time and Voltage will differ based on size of protein of interest.
21. After 2 hours, remove the membrane from the Transfer Apparatus

Blocking and Detecting using the Licor System

1. After transfer, allow the membrane to air dry.
2. Add 10mL of Licor TBS blocking Buffer to the membrane. Allow to gently shake for 1 hour.
3. To the blocking buffer, add 20uL of Tween 20.
4. Immediately add the Primary Antibody to this blocking solution in a concentration of 1:1000-1:5000.
5. Place the membrane with Antibody on a shaker into a 4 degree Environmentally Controlled Room overnight. Shake Gently.
6. Recover the membrane and pour the antibody from the membrane.
7. Add 10mL of 1x TBST to the membrane and shake VIGOROUSLY for 5 minutes. Pour off the 1x TBST. Repeat 3 times.
8. Prepare the secondary antibody solution by mixing 10mL of Licor TBS Blocking Solution, 20uL of Tween 20, 10uL of 10% SDS, and the secondary antibody at a concentration of 1:10,000.

NOTE: the secondary antibody is light sensitive and should be shielded from the light at all times.

9. Pour the secondary antibody from the membrane.
10. Add 10mL of 1x TBST to the membrane and shake VIGOROUSLY for 5 minutes. Pour off the 1x TBST. Repeat 3 times.
11. Add 10 mL of 1x PBS and shake gently for 1 minute. Pour off the PBS.

12. Add 10mL of Deionized Water and shake gently for 30 seconds. Pour off the Water.
13. Allow the membrane to completely dry before imaging.

B.4 Clonogenic Survival Assays for Radioresistance Testing

DAY 1

1. Wipe down the surface of an inverted phase contrast microscope with 70% ethanol and a sterile cover sponge.
2. Examine a cell line (that is currently growing in a T25 flask) using an inverted phase contrast microscope. If the confluency is $\geq 70\%$, the cell line can be used for clonogenics.
3. Warm the appropriate Supplemented-medium, antibiotic-free medium, 0.05% trypsin-EDTA, and Hanks Balanced Salt Solution (HBSS) in a 37° C waterbath.
4. Wipe down all workspaces with 70% Ethanol and a sterile cover sponge. Move the cell line into the Laminar flow biosafety cabinet (the hood).
5. Bring the HBSS and Trypsin into the hood by wiping them down with 70% Ethanol and a sterile cover sponge.
6. Aspirate the medium from the T25 flask using a sterile 9" Pasteur pipette and a vacuum attached to a double set of flasks (500 mL and 1L flasks).
7. Using a Serological Pipette, add 5 mLs of HBSS to the T25 Flask. Let stand for 30 seconds.
8. Aspirate the HBSS using a using a 9" Pasteur pipette, a vacuum, and a vacuum flask.
9. Using a Serological Pipette, add 2-3 mL of Trypsin-EDTA to the Flask. Move the flask into an incubator set at 37° C with a humidity of 5% CO₂
10. Remove the flask from the incubator after 2-5 minutes. While waiting, bring the medium and antibiotic-free medium into the hood after wiping it down with 70% Ethanol and a sterile cover sponge.
11. Gently tap the flask against a hard surface that is sterile and observe using an inverse phase microscope to see if the cells have detached from the surface of the flask. If the cells have attached, move the flask back into the Safety Hood. If they have not, put the flasks back into the incubator for another 2 minutes. Repeat until the cells have detached.
12. To the flask, add 2-3 mL of supplemented-medium using a serological pipette.
13. Pipette the cell suspension into a sterile polystyrene 15 mL Tube
14. Centrifuge the tube at 438 xG for 8 minutes.
15. Bring the tube into the biohazard Safety hood. Remove the supernatant using a 9" Pasteur pipette, a vacuum, and a vacuum flask.

NOTE: be careful as to not disturb the pellet of cells at the bottom of the tube.

16. Resuspend the pellet in 1mL of Antibiotic-Free medium.
17. In a sterile 1mL tube, pipette 20 uL of Trypan Blue.

18. Repeatedly pipette the cell suspension at least 50 times in order to ensure that the cells are distributed evenly throughout the medium.
19. Add 20 uL of this cell suspension to the 1mL tube containing trypan blue.
20. Mix the contents of the 1mL tube thoroughly.
21. Clean a Hemocytometer using 70% Ethanol and a sterile cover sponge.
22. Place a sterile glass coverslip over the middle section of the Hemocytometer.
23. Carefully pipette 10-20 uL trypan blue – Cell Suspension mixture into the well of the Hemocytometer.
24. Using a contrast inverted phase microscope, count the number of live cells in the Hemocytometer matrix.

NOTE: A live cell is categorized as having a green “halo” around the cell under phase. Cells that are not alive will appear dark blue and will be missing the halo.

25. Once the cell count has been determined, calculate how many cells are in the cell suspension (A) by using the following formula:

$$(\text{Cell Count}/2) * 10,000 = \text{Number of cells in the cell suspension (A)}$$

26. Next, calculate how much of that solution you will need to obtain 20,000 cells. This is done using the following formula:

$$(20,000/A) * 1000 = \text{uL of Suspended Cells (X)}$$

27. Once this is determined, obtain 9 sterile 60mm culture dishes and label these with the cell line name, the date, your initials, and either 0 GY (1), 0Gy (2), 0 Gy (3), 2.5 Gy (1), 2.5 Gy (2), 2.5 Gy (3), 5 Gy(1), 5 Gy(2), or 5 Gy (3).
28. Add 10 mL of supplemented medium to a 15mL tube.
29. Add X uL of the cell suspension to this tube and mix thoroughly.
30. Pipette 1 mL of the solution in the 15mL tube to each of the culture dishes. In theory, this should yield 2,000 cells in each culture dish.
31. Add 1mL of supplemented medium to each of the culture dishes
32. Place the culture dishes in an incubator set at 37° C with a humidity of 5% CO₂.

Day 2

33. Carefully clean the inside of a Styrofoam container with 70% Ethanol.
34. Observe the culture dishes under an inverted phase contrast microscope to ensure that the cells have attached.

35. Place the culture dishes in the Styrofoam container, being careful not to spill any of the culture dishes' contents.
36. Seal the container with a piece of red laboratory tape and a biohazard sticker.
37. Transport the container to the location where the culture dishes can be irradiated with the appropriate level of treatment.
38. Once there, irradiate the culture dishes with the appropriate level of irradiation.
39. Transport the culture dishes back and place the culture dishes into an incubator set at 37° C with a humidity of 5% CO₂.

Day 5-14

40. Observe the culture dishes under an inverted phase contrast microscope. Once the colonies for the "0 Gy" treatments appear to have formed ≥ 50 cells, proceed with the following steps.
41. Recover all of the culture dishes related to that cell line from the incubator.
42. Pour off the medium into a collection beaker (size of the beaker is dependent on how many cell lines you will be working with).
43. In each culture dish, add 2 mL of 70% ethanol using a 5mL pipette. Allow the cells to fix at room temperature for 5 minutes.
44. Pour off the 70% ethanol into the collection beaker and add 2 more mL of 70% ethanol to each culture dish. Allow the cells to sit at room temperature for 5 minutes.
45. While waiting, create 7% Giemsa Stain in water. The amount of stain to be made is dependent on how many cell lines you will be working with (100 mLs per cell line).
46. Pour off the 70% ethanol into the collection beaker.
47. Add 2mL of 7% Giemsa to each culture dish using a 5mL serological pipette. Let the culture dishes stand at room temperature for 2-5 minutes.
48. Wash the culture dishes in gently running water to remove excess Giemsa Stain.
49. Allow the culture dishes to dry at room temperature.
50. Once the culture dishes are dried out, count the number of colonies in the culture dish using a dissecting microscope.

NOTE: A surviving colony is defined as having ≥ 50 cells.

51. Record the count for each culture dish in excel and in your notebook.
52. Once all counts have been determined, calculate the plating efficiency using the following formula:

Plating Efficiency (PE) = (# of colonies counted in Untreated control for the treatment) / (# of cells originally seeded)

53. Once plating efficiency has been determined for each treatment, calculate the Surviving Fraction using the following formula:

$$\text{Surviving Fraction (SF)} = (\# \text{ of colonies counted in dish}) / ((\text{Amount of Cells Seeded}) * (\text{PE}/100))$$

54. Graph the mean SF for each treatment using a scatter plot. Perform a Standard Error of the Means for each treatment and see if your results are statistically significant.

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